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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 4 :
A01N 1/02, A61M 37/00
B01D 15/00, G01N 33/543

A1

(11) International Publication Number: WO 89/12390

(43) International Publication Date: 28 December 1989 (28.12.89)

(21) International Application Number: PCT/US88/02067

(22) International Filing Date: 20 June 1988 (20.06.88)

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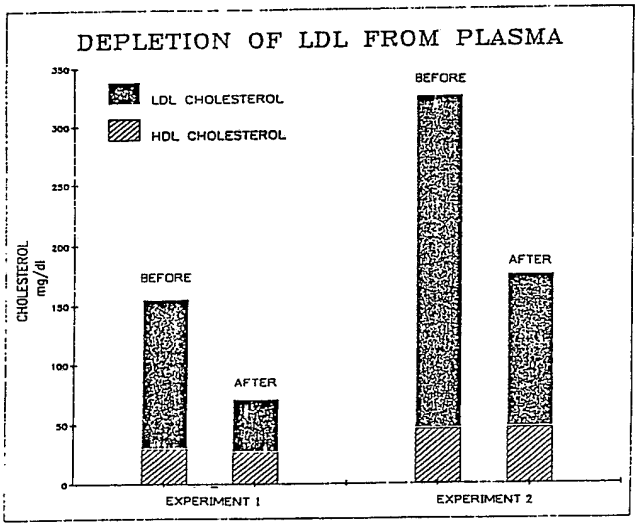
(81) Designated States: AT (European patent), BE (European patent), CH (European patent), DE (European patent), DK, FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), NO, SE (European patent).

Published
With international search report.

(54) Title: BIOCOMPATIBLE, SUBSTANCE-SPECIFIC REAGENTS FOR TREATING PHYSIOLOGICAL FLUIDS

(57) Abstract

The preparation and method of use of biocompatible water-insoluble reagents for removal of substances form physiological fluids is described. The reagent, comprising a carrier substrate with a layer of immobilized binding agent and biocompatibility agent, is produced by contacting an activated carrier substrate concomitantly or sequentially with binding agent and biocompatibility agent. The biocompatibility agent, which comprises an organic molecule with a nucleophilic moiety and a negatively-charged moiety separated by a spacer arm, reacts with the substrate surface through its nucleophilic moiety. The reagent may be incorporated in conventional extracorporeal systems for hemo- or plasmaperfusion to remove substances such as low-density lipoprotein.



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TITLE
BIOCOMPATIBLE, SUBSTANCE-SPECIFIC REAGENTS FOR TREATING PHYSIOLOGICAL FLUIDS

Technical Field

This invention relates to the preparation and use of biocompatible water-insoluble reagents for removal of substances from physiological fluids comprising specific binding ligands and biocompatibility agents immobilized on the surface of a carrier substrate.

Background of the Invention

The selective removal of deleterious substances from blood, blood plasma, or other physiological fluids can provide important therapeutic benefits. For example, high levels of circulating lipids, particularly cholesterol, which is carried in low density lipoprotein (LDL), is a cause of atherosclerosis. Left unchecked, atherosclerosis can lead to serious circulatory system diseases resulting in stroke and/or myocardial infarction. Reduction in the plasma LDL levels is believed to significantly reduce the incidence of such events in high-risk patients [A.M. Gotto, et al Circulation, 69, 1065A-1090A(1984)]. Selective removal of LDL should be an effective method for reducing atherosclerosis. Similarly, the ability to intervene in arthritis by removal of circulating rheumatoid factor, immune related diseases by removal of antibodies, or cancer by removal of malignant cells are being pursued in

medical science. At present, selective depletion plasmapheresis, which involves passage of 2-4 liters of blood or plasma through an extracorporeal device containing the selective adsorbent, represents an attractive therapy.

Clinical use of such treatment modalities, of necessity, brings the physiological fluid into contact with foreign surfaces. In the case of blood and blood plasma, one of the sequelae that can result is activation of the complement cascade which can lead to life threatening anaphylaxis. In practicing such treatment modalities, blood or plasma has been anticoagulated using citrate and/or heparin. Citrate is often employed as much for its ability to interfere with the complement cascade as for its ability to block coagulation. The reliance upon citrate to inhibit complement can be risky as citrate is toxic at doses very close to its dose for use as an anticoagulant. Typically, citrate is employed at the dose where mild toxicity is observed (numbness and tingling of the lips). The toxicity of citrate is due to calcium chelation interferring with normal nerve conduction. The usefulness of extracorporeal devices containing highly selective adsorbing materials is therefore dependent not only on the specificity and affinity of the material, but also its biocompatibility with body tissues and biochemical systems.

Stoffel and Demant [Proc. Nat. Acad. Sci. USA, 78, 611-615 (1981)] describe the application and effectiveness of immunoadsorption chromatography to remove low density lipoprotein from plasma. Utilizing polyclonal anti-swine LDL antibodies covalently

attached to Sepharose CL-4B, swine were treated with an extracorporeal device having a plasma separator and the immunoadsorbent interposed in series in an arteriovenous shunt. Plasma LDL concentration was found to progressively decrease as the duration and flow volume increased in the treatment.

Koren et al. [Biochimica et Biophysica Acta, 876, 202-207 (1986)] disclose the use of a mouse monoclonal anti-human-LDL antibody coupled to an agarose support to remove LDL from plasma samples. No apheresis was performed on any organism.

Parker et al. [Proc. Nat. Acad. Sci. USA, 83, 777-781 (1986)] have treated patients by LDL-pheresis utilizing sheep polyclonal anti-LDL antibodies attached to sepharose. Significant reductions in LDL levels were achieved by the therapy.

European Patent Application 83112042.3 published June 6, 1984 discloses the use of immobilized heparin or dextran sulfate to remove LDL from plasma. The adsorbent utilized comprised a water-insoluble porous hard gel to which a binding ligand was attached. Ligands were attached through reaction with epoxy groups introduced into polymer gels by reaction with epichlorohydrin or a polyoxirane compound, or in the case of inorganic gels, with epoxy groups introduced by reaction with epoxy silane coupling agents. Gels with surface hydroxyl groups were therefore preferred. While a number of selective binding ligands were disclosed, only heparin and dextran sulfate (adsorbents removing numerous plasma proteins) were exemplified as LDL removal systems.

Japanese Patent Application JP60087854 discloses a blood purifying adsorber for removal of malignant substances or cells from blood or plasma. The adsorbent comprises a carrier such as glass, charcoal, cellulose triacetate, and various polymers to which a negatively charged compound and an organic ligand have been attached. The attachment requires the use of strong electrophilic reagents, however, which cannot be utilized in concomitant or subsequent reactions incorporating complex biological binding ligands.

European patent application EP-103184A discloses biospecific polymers carrying immobilized biological agents. The biocompatible polymer support is generally a hydrogel or other wettable polymer to which a biological agent has been covalently bounded. No biocompatibility agents are disclosed which would generally provide protection against adverse reactions in vivo.

Japanese patent application J602239425 discloses the use of solid carriers with attached monoclonal antiapolipoprotein B antibodies to remove LDL from human plasma. No biocompatibility agent was utilized to help protect against adverse reactions with tissues or systems in vivo.

Japanese patent application JP59200655 discloses the use of an adsorbent consisting of an insoluble carrier, preferably hydrophilic, with an attached organic compound containing a negatively charged group. The immobilization of the binding ligand is through the negatively charged group. The application of the adsorbent to remove malignant cells is discussed.

At this time there exists a clear need for an adsorbent reagent which incorporates specific binding ligands and biocompatibility agents to remove pernicious substances from physiological fluids. Such reagents should exhibit both high selectivity and protection against activation of the complement system or blood coagulation. Enhanced biocompatibility of the reagent would enable a reduction in the amounts of anticoagulants administered. The activity of complex biological ligands is highly sensitive to the immobilization technique. A method of preparing such reagents is also required which is mild enough to avoid denaturation, yet flexible enough to permit concomitant or sequential immobilization of binding ligands and biocompatibility agents.

Summary of the Invention

A process for immobilizing both specific binding ligands and biocompatibility agents on a wide variety of carrier substrates has been discovered. The novel reagents produced remove substances from physiological fluids while minimizing adverse reactions such as complement activation. Specifically, one aspect of this invention is a water-insoluble reagent for removal of a substance from physiological fluids comprising (a) a carrier substrate, and immobilized on the surface of said carrier substrate, (b) a binding ligand specific for said substance, and (c) a biocompatibility agent comprising an organic molecule with a nucleophilic moiety and a negatively-charged moiety separated by a spacer arm, said biocompatibility agent immobilized on said surface through reaction with said nucleophilic moiety. Another aspect of this invention involves a process for preparing such a water-insoluble reagent for removal of a substance from physiological fluids comprising the step of contacting and reacting a carrier substrate with an aqueous solution of a binding ligand specific for said substance and biocompatibility agent comprising an organic molecule with a nucleophilic moiety and a negatively-charged moiety separated by a spacer arm. This process can comprise both simultaneous or sequential contacting and reacting of the carrier substrate with the aqueous solutions of binding ligand and biocompatibility agent. Yet another aspect of the invention involves a method of treating a physiological fluid to remove a substance comprising contacting said fluid with a water-insoluble reagent as described above. As a final aspect, the instant invention provides an

apparatus for the extracorporeal treatment of physiological fluids to remove a specific substance comprising means for withdrawing whole blood from a mammal, means for separating plasma from the whole blood, means for treating said plasma including a chamber containing a water-insoluble reagent as described above which will interact with and deplete said substance from the plasma, and means for recombining the substantially substance depleted plasma with the remainder of the whole blood and for returning the recombined whole blood to the mammal.

In the case of LDL removal from blood plasma, preferred reagent and processes would utilize an activated neutral polymer carrier substrate, polyclonal anti-LDL antibody, and a biocompatibility agent with immobilization carried out in two steps at a pH of from about 4 to about 10 controlled by a non-nucleophilic buffer. Most preferred, by virtue of convenience of preparation and/or efficiency of operation is the reagent comprising the immobilization of monoclonal anti-LDL antibody and sulfamic acid on tresyl-sepharose in a one-step concomitant reaction at about pH 8 in potassium phosphate buffer.

Brief Description of the Drawings

Figure 1 is a bar graph representing LDL and HDL plasma concentrations pre- and post-treatment for two experiments utilizing human plasma in vitro.

Detailed Description of the Invention

Preparation of Materials

This invention provides a versatile method for synthesizing novel biocompatible reagents for removal of specific substances from physiological fluids through the immobilization of specific binding ligands and biocompatibility agents on substrate carriers under mild conditions which preserve the activity of sensitive biological ligands. In the context of this disclosure, a number of terms shall be utilized. As used herein, "carrier substrate" is a solid supporting matrix "activated" by well-known coupling chemistries to introduce reactive surface moieties susceptible to nucleophilic attack and displacement by binding ligands or biocompatibility agents to be immobilized on the carrier substrate. "Binding ligand" refers to any substance, or group of substances, which has specific binding affinity for the substance to be removed from the physiological fluid to the exclusion of other substances. The binding ligand may consist of a polyclonal or monoclonal antibody in the form of whole antiserum or ascites fluid, an IgG fraction or as active fragment of such materials. Other specific binding proteins, for example lectins, antigens, or protein A, may be utilized. As used herein, "biocompatibility moiety" refers to a substance which renders the carrier substrate more biocompatible vis-a-vis complement activation. The term

"nucleophilic moiety" refers to chemical groups, for example NH_2, SH or OH, which carry an unshared pair of electrons and generally attack another molecule at a site where the atomic nucleus is poorly shielded by outer electrons. As used herein, the term "negatively charged moiety" refers to chemical groups, for example OSO_3^-, RSO_3^-, RSO_2^-, COO^-, which carry a net negative formal charge. The nucleophilic moiety and negatively charged moiety are contained within the same molecular entity which constitutes a "spacer arm" separating the two functional moieties.

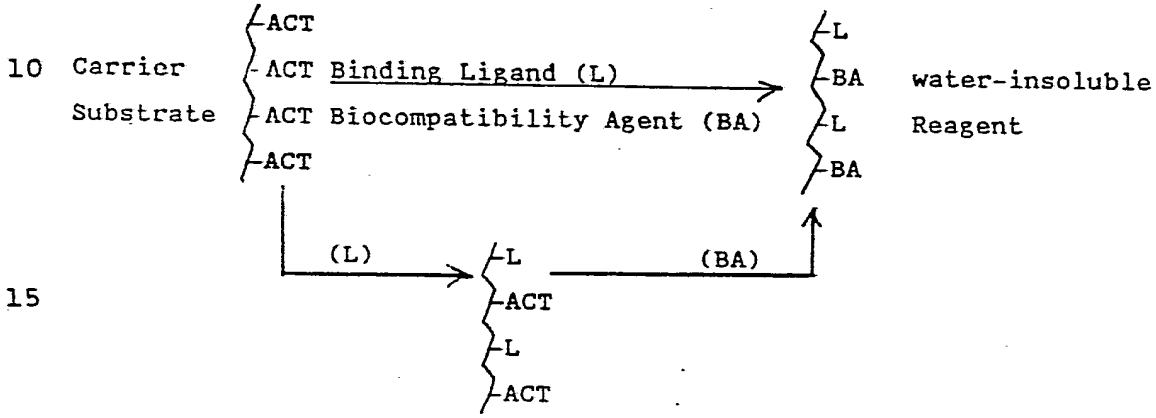
As used herein, the term "neutral polymer" refers to a water insoluble material, either natural or synthetic, that consists of repeating subunits and can be formed into a variety of possible configurations such as flat sheets, fibers, beads, porous beads, membranes, foams, etc. "Non-nucleophilic buffer" refers to any water buffering substance that does not contain a nucleophilic moiety such as R-OH, R-SH, $R-NH_2$, R_2NH, and the like. "Polyclonal antibody" refers to any collection of antibodies directed against a substance where the collection of antibodies directed against a substance arise from many different antibody producing cells. "Monoclonal antibody" refers to any antibody that comes from a single antibody producing cell line.

Carrier Substrate

The water-insoluble reagents of this invention involve the immobilization of binding ligands and biocompatibility agents on activated solid supporting matrices (carrier substrates) utilizing such mild

coupling chemistry that sensitive biological ligands and biocompatibility agents can be reacted concomitantly. The immobilization process is schematically represented in Equation 1 indicating

Equation 1



the possibility of either sequential or simultaneous reaction of the binding ligand (L) and biocompatibility agent(BA). Activation of various solid supporting matrices is achieved using chemistry appropriate for the material to introduce a chemical moiety (ACT) susceptible to nucleophilic attack by chemical groups within the molecules of the binding ligand and the biocompatibility agent.

A variety of materials are suitable as solid supporting materials for the present invention. These materials generally possess the following characteristics: (1) sufficient surface area to effectively trap and remove the target substance due to either the existence of pores or availability in a finely divided state, (2) allow for the flow of physiological fluid over or through the material when constrained in an extracorporeal treatment

apparatus, (3) surface chemical groups which can be activated for subsequent reaction with binding ligand and biocompatibility agent, and (4) stable to biological milieu. Materials fulfilling these criteria include synthetic polymers, natural polymers, silica, alumina and zirconia. The physical geometry of the solid supporting matrix can be as a particle, bead, fiber, hollow fiber, thin sheet, foams, or any other permitting fluid flow over or through the material. Table I lists a variety of commercially available carrier substrates which can be utilized to practice the instant invention. The list is representative since other materials and activations chemistries meeting the above four criteria will be known to those skilled in the art.

TABLE I

CARRIER SUBSTRATES

Carrier Substrate
Affigel 10 (NHS activated agagrose, BioRad Co, Richmond CA)
Affigel 15 (NHS activated agagrose, BioRad Co, Richmond CA)
Tresyl-Sepharose (tresyl activated agarose, Pharmacia Chemical, Upsala, Sweden)
CNBr-Sepharose (CNBr activated agarose, Pharmacia Chemical, Upsala, Sweden)
Reacti-Gel (CDI activated agarose/dextran Pierce Chemical)
Eupergit C (allylglycidyl ether containing terpolymer, Rohn Pharma, Darmstadt Germany)
Epoxy-activated agarose
Epoxy-activated silica
Carboxy Sepharose
Thiol(activated) Sepharose
Trityl-agarose
Oxirane acrylic beads
p-Nitrophenol agarose
Tosyl-agarose
Periodate-treated agarose
Activated CM-cellulose
Activated cellulose
poly-glycidyl methacrylate
PEI-glutaraldehyde activated nylon
PEI-glutaraldehyde activated silica
Glutaraldehyde-activated agarose
Isocyanate-activated agarose
Maleimide-activated agarose
Chloromethylpolystyrene

Activation of supporting matrix materials to produce suitable carrier substrates with surface groups susceptible to nucleophilic attack is carried out using well-known chemical techniques and process reagents. See Scouten, W.H., Affinity Chromatography: Bioselective Adsorption on Inert Matrices, Wiley & Sons, New York, (1981), herein incorporated by reference. In the case of synthetic and natural polymers, activation reagents may include cyanogen halides, epichlorohydrins, polyoxiranes, periodates, polyethyleneimines, or glutaraldehyde. In the case of the most preferred reagent for LDL removal from blood plasma, Sepharose®4B (Pharmacia Fine Chemicals, Uppsala, Sweden; beaded agarose gel) possessing surface hydroxyl groups is reacted with tresyl chloride (2,2,2-trifluoroethanesulfonyl chloride) to produce a surface sulfonate ester (Tresyl-activated Sepharose®4B, Pharmacia Fine Chemicals) as depicted schematically in Equation 2.

Equation 2

$$—CH_2OH + ClSO_2CH_2CF_3 \longrightarrow CH_2OSO_2CH_2CF_3$$

agarose tresylchloride "tresyl-activated" agarose

Such sulfonate esters have been reported to undergo nucleophilic displacement by amines, thiols, hydroxyl, and imidazole groups to produce stable chemical bonds which immobilize various binding ligands [Nilsson, K. and Mosbach, K., Biochem Biophys. Res. Comm., 102, 449-457 (1981) and Methods in Enzymology, 104, 56-69 (1984)].

In the case of an inorganic supporting matrix material including silica, alumina or zirconia, activation may be achieved by reaction with an

epoxysilane such as gamma-glycidoxypropyltrimethoxy-silane, an aminosilane such as gamma-aminopropyl-triethoxysilane, or polyethyleneimine-glutaraldehyde.

Binding Ligand

A wide variety of binding ligands may be utilized with the instant invention depending upon the specific substance to be removed from the physiological fluid. Binding ligands possessing great specificity permit the selective removal of the substance with the least overall impact on the composition of the physiological fluid being treated. A representative list of binding ligands, conjugate substance to be removed, and the disease/disorder to be treated is provided in Table II.

TABLE II

BINDING LIGANDS

Binding Ligand	Conjugate Substance	Disease/Disorder
Any antigen such as	Corresponding antibodies	
Factor VIII or IX	Corresponding antibodies	Factor resistant Hemophilia
HLA antigen	Corresponding antibodies	Transplant rejection
DNA, RNA, nucleotides	Corresponding antibodies	SLE, or other ANA related disease e.g. Sjogren's, scleroderma, etc
Acetylcholine receptor	Corresponding antibodies	Myasthenia Gravis
Blood group antigen, e.g A,B,O	Corresponding antibodies	Transplantation
Tumor antigens	Blocking Factors	Cancer
Xenotypic antigens	Corresponding antibodies	Remove antibodies that neutralize antibody based therapeutics diagnostics
Nervous tissue antigens	Corresponding antibodies	Guillan-Barre Syndrome, MS, etc
Glomerular Basement membrane	Corresponding antibodies	Goodpasture's disease
Anti-LDL antibodies	LDL	Hypercholesterolemia, Other lipid disorders, i.e. Fabry disease
Anti Idiotypic Antibodies	Other antibodies	Autoimmune disease, hyperglobulinemia, transplant rejection, any deleterious antibodies
Protein A, protein G, or Ig binding peptide fragments	Immunoglobulins	Autoimmune disease, hyperglobulinemia, transplant rejection, any deleterious antibodies
Anti-endotoxin antibodies	Endotoxin	Septic shock
Anti-drug or toxin antibodies	drugs, toxins, psychoactive cpds.	Drug overdose, poisoning, psychotic syndromes
Anti-B, or t-Cell antibodies	B, or T cells	Lymphoma or leukemia, bone marrow transplant
C1q, or other complement component	Immune complexes	Autoimmune diseases
Anti-killer cell antibodies, or cell specific ligands eg IL-2	Functional white cells	Adoptive immunotherapy

It is to be understood that the binding ligands of the present invention are not limited to those set forth in Table II which are meant to be illustrative. These binding ligands may be used alone or in mixtures if appropriate.

In the case of LDL removal from blood plasma, highly specific monoclonal antibodies are most preferred as binding ligands. An anti-LDL antibody was prepared using a standard monoclonal antibody immunization and fusion protocol [Vernon T. Oi and Leonard A. Herzenberg. Selected Methods in Cellular Immunology, Eds. B. Mishell and S. Shiigi, 1980, pp 351-372] Balb/CJ mice were immunized with purified human LDL with and without complete Freund's adjuvant. The mice were boosted at regular intervals and bled at various time points for several weeks following the immunizations. Serum samples were tested in an ELISA to determine the amount of anti-LDL antibodies present in the immunized mice versus the non-immunized Balb/CJ.

Several months following the initial immunization, the mice were boosted with antigen and the first fusion was performed. A spleen cell suspension from a primed mouse was fused with SP2/O myeloma cells, mixed with an irradiated feeder spleen cell suspension, and plated out. The clones were fed at various intervals and observed for growth.

Clones were selected and assayed to determine the amount of IgG being produced, and each clone was isotyped. Clones were tested regularly to monitor IgG production. At a later date, cell supernates from selected clones were tested in a LDL ELISA to

determine specific activity of each selected clone. The most productive clones were selected and frozen in liquid nitrogen. Several of the better clones were subcloned and assayed to ensure that a monoclonal anti-LDL was being produced and to try and increase IgG production and LDL specificity. All of the clones producing specific anti-LDL monoclonal antibody were further assayed for other factors, such as stability, antibody production, affinity, etc, that would select the best clones for use in this invention.

Biocompatibility Agent

A number of bifunctional chemical entities incorporating both a nucleophilic moiety, for reaction with the carrier substrate, and a negatively charged moiety separated by a spacer arm are contemplated as biocompatibility agents of the instant invention. The negatively charged moiety is tethered at the surface following reaction of the nucleophilic moiety with the carrier substrate to immobilize the biocompatibility agent.

Many combinations of nucleophilic moiety and negatively charged moiety are contemplated by the present invention. Nucleophilic moieties including for example RNH_2, RSH, ROH, or R_2NH can be utilized. Negatively charged moieties including OSO_3^-, RSO_3^-, RSO_2^-, CO_2^-, are suitable. A representative list of biocompatibility agents is provided in Table III.

TABLE III

BIOCOMPATIBILITY AGENTS

Nucleophilic Moiety	Negatively Charged Moiety	Specific Examples
Amino	Sulfate	Sulfamic Acid Taurine Sulfanilic Acid
Amino	Carboxylate	All amino acids e.g. glycine, aspartic acid, cysteine
Hydroxy	Sulfate	Isethionic Acid Hydroxybenzenesulfonic Acid Glucose sulfate
Hydroxy	Carboxylate	Lactic Acid Uronic Acids, e.g. Glucuronic Acid Glyconic Acids, e.g. Gluconic Acid
Thiol	Sulfate	2-Mercaptoethanesulfonic Acid
Thiol	Carboxylate	Mercaptoacetic Acid Thiosalicyclic Acid Thiolactic Acid

It is apparent from Table III that a number of bifunctional molecules with separated nucleophilic and negatively charged moieties can function as biocompatibility agents. It will be appreciated that the molecular structure into which such moieties are incorporated serves as a spacer arm to separate the moieties and permit independent function, i.e. reaction with carrier substrate and protection against adverse biological interactions. As such the spacer arm is not critical and one skilled in the art can select various molecular structures as spacer arms into which to incorporate the functional moieties.

Water-Insoluble Reagent

Binding ligands and biocompatibility agents are immobilized on the surface of the carrier substrate by nucleophilic displacement reactions involving a nucleophile of the binding ligand or biocompatibility agent and the activated moiety on the carrier substrate. In the case of protein binding ligands such as antibodies, the numerous amino groups within the antibody serve as nucleophiles for convenient reaction. The processes of the instant invention are schematically represented by Equation 1.

Simultaneous attachment of a Binding ligand and Biocompatibility Agent to the Carrier Substrate: In this process, the water-soluble reagent is prepared by adding the carrier substrate to a reaction solution containing the biocompatibility agent and binding ligand or vice versa. The carrier substrate, such as those listed in Table I, can be added as a dry material, or wetted by a suitable buffer. The reaction solution contains binding ligand at a

concentration of from about 10 mg/ml to about 100 mg/ml and biocompatibility agent at a concentration of from about 0.01 M to about 5 M. The reaction solution can also be buffered with any non-nucleophilic buffer, such as phosphate, at a buffer concentration of from about 0.01 M to about 5 M. The pH range of the reaction solution can vary from about 4 to about 10. After combining the reaction solution with the carrier substrate, the resultant mixture is agitated for 30 minutes or longer, up to several days if required, at any temperature that does not freeze the mixture or deform the carrier substrate, typically 4 C to 45 C. After coupling, the reaction solution is separated from the water-soluble reagent and rinsed to remove unreacted biocompatibility agent and binding ligand. The resultant derivatized carrier substrate comprises the water-insoluble reagent.

Stepwise attachment of a Binding Ligand and Biocompatibility Agent to the Carrier Substrate: In this process, the water-insoluble reagent is prepared by first reacting the carrier substrate with binding ligand and then adding biocompatibility agent to the reaction solution in a second step. This is the preferred method in cases where the binding ligand and biocompatibility agent are not compatible in the same solution, or if the reaction conditions for immobilization of binding ligand and biocompatibility agent are sufficiently different. The ranges for the reaction parameters in stepwise reactions are the same as for the simultaneous reactions described above.

EXAMPLE 1

Anti-LDL Water-Insoluble Reagent preparation using Simultaneous Attachment of Anti-LDL Monoclonal Antibody CLL1 (binding ligand) and Sulfamic Acid (biocompatibility agent): Tresyl-Sepharose (carrier

substrate; 35 gm) was sterilized by washing with acetone and filtering through a sterile sintered glass funnel. The acetone washed bead was then transferred to a sterile roller bottle (to aid transfer it is recommended that the bead be slightly wetted with acetone). To the roller bottle was added 105 ml of the reaction solution containing monoclonal antibody CLL1 (3.0mg/mL), sulfamic acid (0.25 M), and potassium phosphate (0.25 M) at pH 8.0. The slurry was gently agitated overnight (16 hr) at ambient temperature. After overnight agitation, the slurry was filtered using a sintered glass funnel and the combined sorbent washed with 1 L of citric acid (pH 3.0), followed by 1 L of buffered saline (pH 7.1). The filtrate was collected for reprocessing and reuse. The sterile anti-LDL sorbent was transferred to a sterile container where merthiolate was added (final concentration 0.1%) as a preservative of sterility until further use.

EXAMPLE 2

Anti-LDL Water-Insoluble Reagent preparation using Stepwise Attachment of Anti-LDL Monoclonal Antibody CLL1 (binding ligand) and Sulfamic Acid (biocompatibility agent): Tresyl-Sepharose (carrier substrate; 0.5 gm) was sterilized by washing with acetone and filtering through a sterile sintered glass funnel. The acetone washed bead was then transferred to a sterile centrifuge tube (to aid transfer it is recommended that the bead be slightly wetted with acetone). To the centrifuge tube was added 5 mL of the reaction solution containing monoclonal antibody CLL1 (3.0 mg/mL), and sodium bicarbonate (0.10 M) at pH 8.0. The slurry was gently agitated for 4 hours at ambient temperature. The antibody solution was

separated from the carrier substrate by centrifugation at 1000 x g for 10 min and decanting the unreacted antibody. Sulfamic acid (0.20 M) in sodium carbonate buffer (0.050 M), pH 9.0, was then added and the mixture agitated overnight (16 hr) at ambient temperature. After overnight agitation, the slurry was filtered using a sintered glass funnel and the anti-LDL water-insoluble reagent washed with 1 L of citric acid (pH 3.0), followed by 1 L of buffered saline (pH 7.1). The decanted supernatant and filtrate was collected for reprocessing and reuse. The sterile anti-LDL water-insoluble reagent slurry was transferred to a sterile container where merthiolate was added (final concentration 0.1%) as a preservative of sterility until further use.

Utility

The water-insoluble reagent described above can be used to selectively remove substances from physiological fluids which are bound by the binding ligand. The water-insoluble reagent would be confined in a container housing that would allow the flow of the physiological fluid through the water-insoluble reagent. If the physiological fluid were blood or blood plasma, the water-insoluble reagent would be used to remove from the blood or plasma a substance that is associated with a disease or disease process. This would be the case in a number of diseases where it is known or suspected that the removal of the disease associated substance would be beneficial. These include auto-immune diseases, metabolic disorders, and others. A more complete list of the diseases that could be amenable to this therapy, the substance(s) to be removed, and the possible ligands

that could accomplish this are shown in Table II. The water-insoluble reagent listed in Table I, one or more of the binding ligands listed in Table II, and one or more of the biocompatibility agents listed in Table III.

To illustrate the use of the invention for a therapeutic purpose, a volume or amount of a reagent as described above would be contained in a housing that would allow for the flow of blood or plasma. Anticoagulated blood or plasma from a patient would be pumped through the housing containing the water-insoluble reagent and then returned to the patient. The blood or plasma treated in this way would thus be unaffected except for the depletion of the desired substance bound by the binding ligand in the water-insoluble reagent. There are two parameters by which the performance or utility of the system can be evaluated: 1) the effectiveness of the binding ligand, and; 2) the effectiveness of the biocompatibility agent.

The effectiveness of the binding ligand can be determined by measuring the amount of a substance in the physiological fluid before and after passage over the water-insoluble reagent. The amount of this substance depleted is a measure of the performance of the reagent. The assay system for determining the amount of the substance removed is, of course, dependent upon the substance. To further illustrate how this would be accomplished, in the case of LDL, plasma that has been anticoagulated would be perfused over an anti-LDL water-insoluble reagent. Samples of the plasma, before and after perfusion over the combined sorbent, would be collected and assayed. Plasma samples would then assayed for total

cholesterol and HDL. The LDL levels are easily calculated from the HDL and total cholesterol values as the total cholesterol level of plasma is presumed to be the sum of HDL and LDL.

The total cholesterol determinations would be performed by standard clinical laboratory procedures. This procedure is a quantitative enzymatic reaction using cholesterol oxidase following chemical saponification of the cholesterol esters. The by-product of the cholesterol oxidase (H_2O_2) is coupled with peroxidase and a colorimetric substrate. This reaction leads to a colored end product which can be measured using a spectrophotometer [Stein, E. A., In, Textbook of Clinical Chemistry, Tietz, N. W., Ed., WB Saunders, Philadelphia, 1986, pp 879-886, 1818, and 1829].

The HDL determinations are performed by first precipitating the LDL, by use of a precipitating reagent, and assaying the supernate via the above total cholesterol procedure.

The effectiveness of the biocompatibility agent can be determined by measuring the amount of complement activation in the plasma as a result of exposure to the combined sorbent. Attenuation of the complement activation as compared to the untreated polymer carrier is the demonstration of performance of the biocompatibility agent. Activation of the complement cascade by a foreign substance occurs via activation of the alternate pathway. The components of the complement cascade that are quantitated to determine the amount of activation are C_{3a} and C_{5a}. C_{3a} (or C_{5a}) is determined by a known RIA

technique [see Chenoweth, D.E. and Hugli, T.E., J. Biol. Chem 250, 8293(1975), herein incorporated by reference] wherein antibodies specific to human C_{3a} (or C_{5a}) are allowed to react with a heparin anticoagulated plasma sample that has been exposed to the water-insoluble reagent. Use of other anticoagulations such as citrate, can significantly alter complement activation. The degree to which the specific antibody is bound to C_{3a} (or C_{5a}) is determined by a radioisotope probe. The amount of C_{3a} (or C_{5a}) in a plasma sample can then be calculated from the measured amount of radioactivity. The amounts of C_{3a} and C_{5a} are summed to give a value representative of the total invitro complement activation of the tested material.

The water-insoluble reagent can be contained in various shaped housings depending upon the physical form of the solid support material or the application. The shape of the apparatus employed is not critical to this disclosure, any apparatus that restrains the water-insoluble reagent and allows for flow of the physiological fluid can be used.

Utilizing the above techniques, the effectiveness of the water-insoluble reagent to remove LDL from plasma can be demonstrated by measuring the LDL levels before and after perfusion of a plasma sample over the water-insoluble reagent. Employing the water-insoluble reagent of Example 1 comprising anti-LDL monoclonal antibody CLL1 (binding ligand) and sulfamic acid (biocompatibility agent) immobilized on tresyl Sepharose, demonstration of LDL binding to the immunosorbent and depletion of LDL from plasma is illustrated by the two experiments shown in Figure 1.

The values for LDL cholesterol (solid bars) and HDL cholesterol (hatched bars; total cholesterol is the sum of HDL and LDL) before and after plasma treatment by the immunosorbent are presented. The immunosorbent effectively depletes LDL from plasma whether a high cholesterol (>300 mg/dl) or a low cholesterol (155 mg/dl) plasma is used. Also note that HDL levels are unaffected by the treatment with the LDL immunosorbent, attesting to the specificity of the water-insoluble reagents.

The effectiveness of the biocompatibility agent to attenuate complement activation in the plasma as compared to the untreated polymer carrier is shown in Table IV.

Table IV

Material	C_{3a} µg/ml	C_{5a} µg/ml	Total Activation
Untreated Sepharose	82	19	101
Hydrolized tresyl-Sepharose	56	15	71
Tris-Sepharose	60	3	63
Sulfanilic-Sepharose	44	4	48
Sulfamic-Sepharose	43	4	47

As mentioned previously, total complement activation is the sum of C_{3a} and C_{5a} and is expressed as µg/ml in the test plasma. Sulfanilic and sulfamic treatment result in the use of a biocompatibility agent from Table III and a significant reduction in the complement activation of that material is seen. Treatment with Tris (tris(hydroxymethyl)aminomethane, Sigma Chemical Company)) or water hydrolyzed

tresyl-Sepharose does not incorporate the negatively charged moiety onto the surface and does not confer the protection obtained from the use of the biocompatibility agent.

A combination sorbent prepared with a binding ligand and a biocompatibility agent confers both binding and biocompatibility properties to the water-insoluble reagent. Specifically, the water-insoluble reagent prepared using the anti-LDL antibody CLL1 and sulfamic acid (Example 1) demonstrate the combined effect; the ability of the biocompatibility agent to attenuate complement, and the ability of the binding ligand to bind and remove LDL. The results are shown in Table V which lists the amount of LDL bound in mg per ml of water-insoluble reagent as well as the associated complement activation.

TABLE V

REAGENT CHARACTERISTICS

Material	Amount LDL Bound, mg/ml	Complement Activation
Untreated Sepharose	0	143
Sepharose-CLL1-Tris	4.0	72
Sepharose-CLL1-Sulfamic Acid	4.0	53
Blank	N/A	5

EXAMPLE 3

Use of Anti-LDL Combined Sorbent to Remove LDL from Plasma; The anti-LDL reagent, as described above, (1.5 mL) was packed into a 1.0 x 10 cm column containing a fritted disc at the bottom to retain the water-insoluble reagent material. The packed reagent was washed with several bed volumes of phosphate buffered saline at pH 7.4 to remove the merthiolate. Citrated plasma (9.0 mL) was then recirculated through the column for 1 hr. Samples of the plasma before and after recirculation were assayed to determine the amount of removal of LDL or other constituents from the plasma. The removal of LDL from the plasma is illustrated in Figure 1. Quantitation of the lipoprotein levels (HDL and LDL) in plasma is achieved by measuring the cholesterol content of the respective lipoprotein. The values of LDL cholesterol (solid bars) and HDL cholesterol (hatched bars; total cholesterol is the sum of HDL and LDL) before and after treatment of the plasma by the anti-LDL water-insoluble reagent are presented. In the studies

depicted in Figure 1, two plasma samples were perfused over the reagent. Note that the reagent removes LDL effectively whether a high cholesterol (>300 mg/dl) or a low cholesterol (155 mg/dl) plasma is used. Table VI shows the effect of the plasma recirculation over the water-soluble reagent on the other plasma components before and after recirculation. There were no significant changes in the levels of the other plasma constituents.

TABLE VI
SUMMARY OF TESTING FOR CHANGES IN PLASMA CONSTITUENTS

TESTS	UNITS	EXPERIMENT 1 Before	EXPERIMENT 1 After	EXPERIMENT 2 Before	EXPERIMENT 2 After
LDH	u/l	150.0	137.0	120.0	108.0
SGOT	u/l	20.0	28.0	26.0	35.0
SGPT	u/l	14.0	13.0	30.0	16.0
BILIRUBIN	mg/dl	0.6	0.3	0.4	0.2
TOTAL PROTEIN	gm/dl	6.0	5.9	6.1	5.5
ALBUMIN	gm/dl	3.9	3.7	3.9	3.6
GLOBULIN	gm/dl	2.1	2.2	2.2	1.9
SODIUM	meq/l	155.0	158.0	153.0	144.0
POTASSIUM	meq/l	3.8	4.5	3.6	3.3
CHLORIDE	meq/l	84.0	93.0	77.0	87.0
PHOSPHOROUS	mg/dl	2.7	4.6	2.1	3.1
URIC ACID	mg/dl	4.3	3.4	4.2	3.8
GLUCOSE	mg/dl	83.0	74.0	102.0	93.0
CREATININE	mg/dl	1.0	1.0	0.8	0.9
UREA	mg/dl	17.0	16.0	8.0	7.0
ALKALINE PHOSPHATASE	u/l	59.0	48.0	68.0	65.0
TRIGLYCERIDES	mg/dl	111.0	141.0	179.0	137.0
CHOLESTEROL	mg/dl	155.0	70.0	325.0	175.0
HDL	mg/dl	30.0	27.0	46.0	47.0
LDL	mg/dl	105.0	20.0	248.0	103.0
ALPHA ANTI-TRYPSIN	mg/dl	170.0	166.0	N/A	178.0

TABLE VI (CONTINUED)
SUMMARY OF TESTING FOR CHANGES IN PLASMA CONSTITUENTS

TESTS	UNITS	EXPERIMENT 3 Before	EXPERIMENT 3 After	EXPERIMENT 4 Before	EXPERIMENT 4 After
LDH	u/l	66.0	63.0	45.0	53.0
SGOT	u/l	13.0	14.0	21.0	4.0
SGPT	u/l	17.0	16.0	11.0	6.0
BILIRUBIN	mg/dl	0.1	0.1	0.2	0.1
TOTAL PROTEIN	gm/dl	5.2	5.0	5.3	4.9
ALBUMIN	gm/dl	3.4	3.3	3.4	3.0
GLOBULIN	gm/dl	1.8	1.6	1.9	1.9
SODIUM	meq/l	160.0	160.0	160.0	160.0
POTASSIUM	meq/l	3.5	3.4	3.3	3.2
CHLORIDE	meq/l	111.0	113.0	112.0	129.0
PHOSPHOROUS	mg/dl	4.9	4.8	4.9	6.8
URIC ACID	mg/dl	5.0	4.8	5.7	4.8
GLUCOSE	mg/dl	119.0	115.0	54.0	68.0
CREATININE	mg/dl	9.7	9.4	10.9	9.7
UREA	mg/dl	58.0	57.0	59.0	49.0
ALKALINE PHOSPHATASE	u/l	45.0	42.0	36.0	30.0
TRIGLYCERIDES	mg/dl	124.0	105.0	91.0	70.0
CHOLESTEROL	mg/dl	156.0	102.0	140.0	100.0
HDL	mg/dl	24.0	25.0	21.0	20.0
LDL	mg/dl	132.1	77.0	120.0	80.0
ALPHA ANTI-TRYPSIN	mg/dl	157.0	150.0	N/A	N/A

It should be understood that the detailed description and specific examples provided above, while indicating preferred embodiments of the invention, are given by way of illustration only. From this disclosure one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions.

WHAT IS CLAIMED IS:

1. A water-insoluble reagent for removal of a substance from physiological fluids comprising (a) a carrier substrate, and immobilized on the surface of said carrier substrate, (b) a binding ligand specific for said substance, and (c) a biocompatibility agent comprising an organic molecule with a nucleophilic moiety and negatively-charged moiety separated by a spacer arm, said biocompatibility agent immobilized on said surface through reaction with said nucleophilic moiety.

2. A water-insoluble reagent of Claim 1 wherein said carrier substrate is a neutral polymer.

3. A water-insoluble reagent of Claim 2 wherein said neutral polymer is selected from the group consisting of Affigel 10, Affigel 15, Tresyl-Sepharose, CNBr-Sepharose, Reacti-Gel (CDI activated agarose/dextran), Eupergit C, epoxy-activated Sepharose, epoxy-activated silica, carboxy Sepharose, Thiol(activated) Sepharose, trityl-agarose, oxirane acrylic beads, p-nitrophenol agarose, tosyl-agarose, periodate-treated agarose, activated CM-cellulose, activated cellulose, poly-glycidyl methacrylate, PEI-glutaraldehyde activated nylon, PEI-glutaraldehyde activated silica, glutaraldehyde-activated agarose, isocyanate-activated agarose, maleimide-activated agarose, and chloromethylpolystyrene.

4. A water-insoluble reagent of Claim 3 wherein said neutral polymer is tresyl-Sepharose.

5. A water-insoluble reagent of Claim 1 wherein said binding ligand is selected from the group consisting of a monoclonal antibody, a polyclonal antibody, hormone-receptor, lectin, antigen and vitamin binding protein.

6. A water-insoluble reagent of Claim 5 wherein said binding ligand is a monoclonal antibody specific for the substance to be removed from a physiological fluid.

7. A water-insoluble reagent of Claim 6 wherein said monoclonal antibody is specific for low density lipoprotein.

8. A water-insoluble reagent of Claim 1 wherein said biocompatibility agent comprises an organic molecule with said negatively-charged moiety selected from the group consisting of OSO_3^-, RSO_3^-, RSO_2^-, and COO^-.

9. A water-insoluble reagent of Claim 1 wherein said biocompatibility agent comprises an organic molecule with said nucleophilic moiety selected from the group consisting of NH_2, SH, and OH.

10. A water-insoluble reagent of Claim 1 wherein said biocompatibility agent is selected from the group consisting of sulfamic acid, taurine, sulfanilic acid, glycine, aspartate, cysteine, isethionic acid, Hydroxybenzenesulfonic Acid, glucose sulfate, Lactic Acid, glucuronic acid, gluconic acid, 2-mercaptoethane-sulfonic Acid, Mercaptoacetic Acid, Thiosalicylic Acid, Thiolactic Acid.

11. A water-insoluble reagent of Claim 10 wherein said biocompatibility agent is sulfamic acid.

12. A process for preparing a water-insoluble reagent for removal of a substance from physiological fluids comprising the step of contacting and reacting a carrier substrate with an aqueous solution of a binding ligand specific for said substance and a biocompatibility agent comprising an organic molecule with a nucleophilic moiety and a negatively-charged moiety separated by a spacer arm.

13. The process of Claim 12 wherein the concentration of said binding ligand is from about 10 mg/ml to about 100 mg/ml and the concentration of said biocompatibility agent is from about 0.01 M to about 5.0M.

14. The process of Claim 13 wherein the pH is controlled with a non-nucleophilic buffer in the range of from about 4 to about 10.

15. A process for preparing a water-insoluble reagent for removal of a substance from physiological fluids comprising the steps: (a) contacting and reacting a carrier substrate with an aqueous solution of binding ligand specific for said substance, and (b) contacting and reacting the product of step (a) with an aqueous solution of a biocompatibility agent comprising an organic molecule with a nucleophilic moiety and a negatively-charged moiety separated by a spacer arm to produce said water-insoluble reagent.

16. The process of Claim 15 wherein the concentration of said binding ligand is from about 10 mg/ml to about 100 mg/ml and the concentration of said biocompatibility agent is from about 0.01M to about 5.0M.

17. The process of Claim 15 wherein the pH is controlled with a non-nucleophilic buffer in the range of from about 4 to about 10.

18. A method of treating a physiological fluid to remove a substance comprising contacting said fluid with a water-insoluble reagent of Claim 1.

19. The method of Claim 18 wherein said physiological fluid is mammalian plasma.

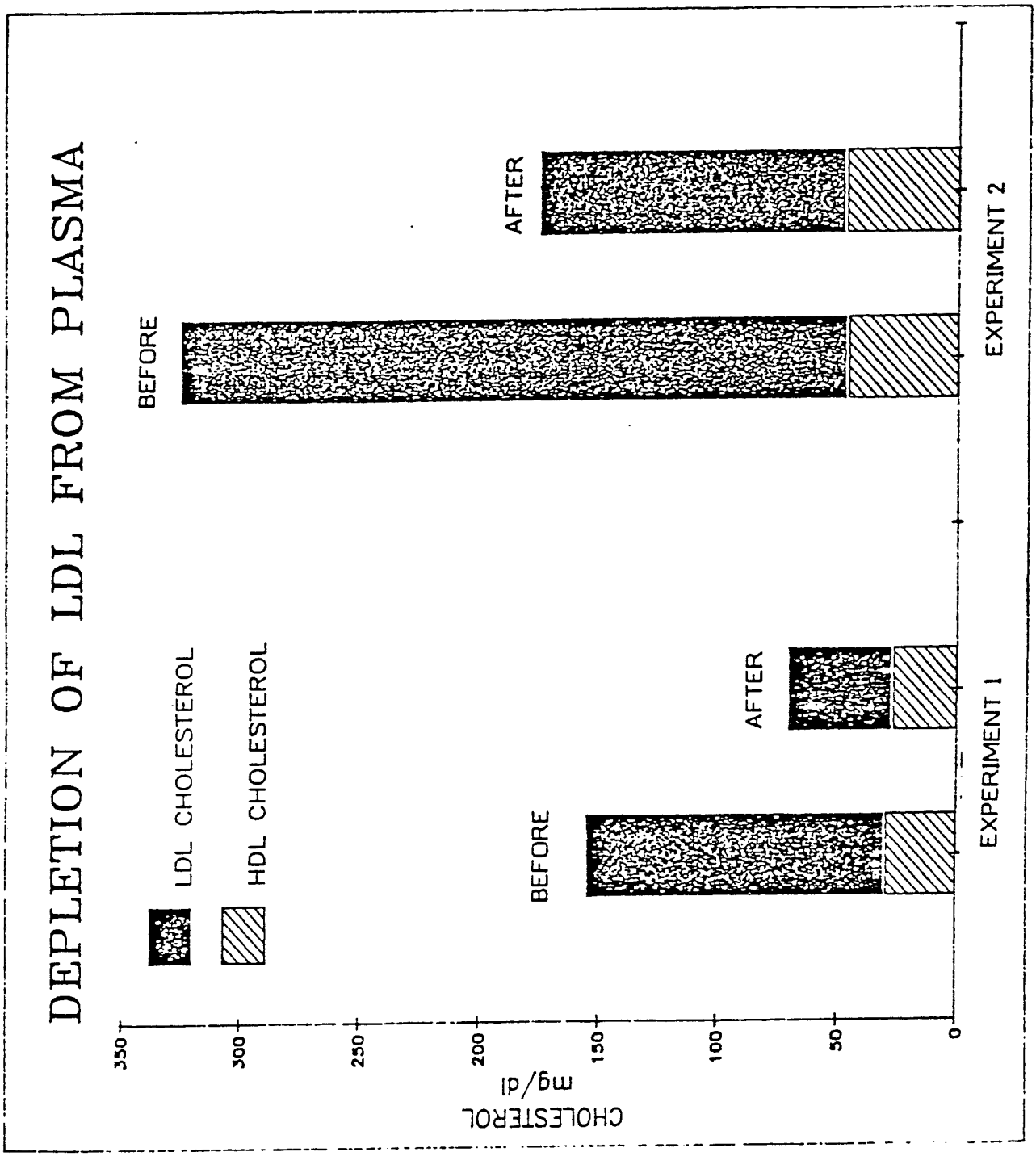
20. The method of Claim 18 wherein said substance is low density lipoproteins.

21. An apparatus for the extracorporeal physiological fluids to remove a specific substance treatment of comprising means for withdrawing whole

blood from a mammal, means for separating plasma from the whole blood, means for treating said plasma including a chamber containing a water-insoluble reagent of Claim 1 which will interact with and deplete said substance from the plasma, and means for recombining the substantially substance depleted plasma with the remainder of the whole blood and for returning the recombined whole blood to the mammal.

22. The apparatus of Claim 21 wherein the water insoluble reagent comprises Tresyl-Sepharose having monoclonal antibody for low density lipoprotein and sulfamic acid immobilized on the surface of said Tresyl-Sepharose.

FIGURE 1


DEPLETION OF LDL FROM PLASMA
LDL CHOLESTEROL
HDL CHOLESTEROL
BEFORE
AFTER
BEFORE
AFTER
EXPERIMENT 1
EXPERIMENT 2
CHOLESTEROL
mg/dl
350
300
250
200
150
100
50
0

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US88/02067

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

INT CL. (4): A01N 1/02; A61M 37/00; B01D 15/00; G01N 33/543
US. CL.: 210/679; 427/2; 436/518,527,530,531,824,825; 530/413; 604/5,6

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S. CL	210/434,679; 427/2; 436/518,527,529, 530, 531, 824, 825; 530/413; 604/5,6

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

APS SEARCH:
S Sulfamic and (Antigdagula? or Extrcoporeal or plasmapheresis)

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	US, A, 4,627,915 (KURODA ET AL.) 9 December 1986. See col. 4, lines 52-65; col. 7, lines 7-13; col. 7, line 64 - col. 8, line 25; col. 9, line 51 - col. 10, line 37; col. 16, lines 12-20.	1-3, 8-19,21
A	US, A, 4,693,985 (DEGEN ET AL.) 15 September 1987. See col. 3, lines 22-54.	18,19
A	US, A, 4,737,544 (McCAIN ET AL.) 12 April 1988. See summary.	21
Y	JA, A, 59-0,189,859 (ASHI CHEMICAL IND. KK) 27 October 1984. See Derwent Abstract.	1-21

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
15 September 1988	09 NOV 1988
International Searching Authority	**Signature of Authorized Officer**
ISA/US	David A. Saunders DAVID A. SAUNDERS

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X Y	JA, A 60-0,087,854 (ASHI-CHEMICAL IND. KK) 17 May 1985. See entire document.	1-3,5, 8-21 4,6,7, 22
Y	JA, A, 60-0,239,425 (TEIJIN KK) 28 November 1985. See Derwent Abstract	6,7,20, 22
Y	Biochimica ET Biophysica Acta, Vol. 876, No., Issued 21 March 1986, (Amsterdam, NL) KOREN ET AL., "Characterization of a monoclonal antibody that binds equally to all aplolipoprotein B. II. isolation of apolipoprotein and lipoprotein forms of human plasma apolipoprotein B. containing lipoproteins from human plasma", pp. 101-107. See abstract.	6,7,20, 22
Y	Biochem. Biophys. Research Commun., Vol. 102, No. 1, issued 16 September 1981 (New York, NY, USA) NILSSON ET AL, "Immobilization of enzymes and affinity ligands to various hydroxyl group carrying supports using highly reactive sulfonyl chlorides" pp. 449-457. See pp. 449-450.	4,22
Y	Proc. Natl. Acad. Sci. USA, Vol. 78, No. 1., issued January 1981 (Washington, D.C. USA) STOFFEL ET AL., Selective Removal of apolipoprotein B-containing serum lipoproteins from blood plasma". pp 611-615. See abstract.	6,7,20, 22
Y	Proc. Natl. Acad. Sci. USA, Vol. 83, No. 2, issued February 1986 (Washington D.C., USA) PARKER ET AL., "Plasma high density lipoprotein is increased in man when low density lipoprotein (LDL) is lowered by LDL-pheresis". pp. 777-781. See abstract.	20,22